PATENT ABSTRACTS OF JAPAN

(11)Publication number : 08-163982
(43)Date of publication of application : 25.06.1996

(51)Int.Cl. C12N 5/06
A61K 38/00
A61K 38/00
G01N 33/53
//(C12N 5/06
C12R 1:91)

(21)Application number : 06-298813
(22)Date of filing : 08.11.1994

(71)Applicant : CHUGAI PHARMACEUT CO LTD
(72)Inventor : TOMONAGA MASAO
KURIYAMA KAZUTAKA
MIYAZAKI TAIJI
HIGUCHI MASATO

(30)Priority
Priority number : 06272823 Priority date : 12.10.1994 Priority country : JP

(54) ERYTHROPOIETIN-DEPENDENT HUMAN CELL LINE

(57)Abstract:
PURPOSE: To obtain a human cell line subject to proliferation specifically dependently on erythropoietin, thus useful for e.g. erythropoietin activity screening operations to test its cell proliferation or differentiating induction by addition of a compound which may have erythropoietin activity.
CONSTITUTION: Bone marrow fluid is taken from patient(s) with initial erythroblast leukemia, the myeloblast in the bone marrow fluid is cultured in an erythropoietin-contg. medium, and the resultant cell line formed a colony is taken, and then further cultured in a medium containing both erythropoietin and bovine fetus serum, thus obtaining the objective new human cell line AS-E2 (FIRM BP-4819) capable of subculture and subject to proliferation specifically dependently on erythropoietin. This cell line can be subjected to erythropoietin activity in vitro screening to test its cell proliferation or differentiating induction by its culture in a medium containing a compound supposed to have erythropoietin activity.

LEGAL STATUS

[Date of request for examination] 22.05.2001

[Date of sending the examiner's decision of rejection]

[Kind of final disposal of application other than the examiner's decision of rejection or application converted registration]

[Date of final disposal for application]

[Patent number]	3696274
[Date of registration]	08.07.2005
[Number of appeal against examiner's decision of rejection]	
[Date of requesting appeal against examiner's decision of rejection]	
[Date of extinction of right]	

* NOTICES *

1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

CLAIMS

[Claim(s)]
[Claim 1] The increased human cell stock which depends for on erythropoietin specifically.
[Claim 2] The cell strain according to claim 1 which is an initial erythroblast leukemia patient's bone marrow cell origin.
[Claim 3] The cell strain according to claim 1 which is AS-E2 (the National Institute of Bioscience and Human-Technology patent microorganism deposition pin center,large, trust number FIRM:BP - 4819).
[Claim 4] The in vitro screening procedure of the erythropoietin activity which consists of adding the compound imagined to have erythropoietin activity to a cell strain according to claim 1, and examining cell proliferation or differentiation inducing.

[Translation done.]

* NOTICES *

1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Industrial Application] This invention relates to the screening procedure of erythropoietin activity using the increased human cell stock which depends for specifically [it is still more detailed and] to erythropoietin, and this cell strain about an erythropoietin dependency human cell stock.
[0002]
[Description of the Prior Art] Erythropoietin (it may be hereafter written as Epo) is a glycoprotein which controls cell proliferation differentiation of the erythrocyte in a higher animal (for example, Carnot et al., Compt.Rend.143:384, 1986 reference). Although an erythrocyte is produced by maturation and differentiation of erythroblast in bone marrow, erythropoietin is cytokine which acts on an undifferentiated precursor cell and guides the differentiation to the erythrocyte. From such an operation, erythropoietin is greatly used as a clinical treatment medicine of ischemia, especially renal anemia in recent years.
[0003] Most erythropoietin currently used in the current clinical field is the various recombinant obtained by gene modification technology. Moreover, in recently, it is not recombination protein itself [such]. With low-molecular, more erythropoietin activity To replace with the compound which it has is tried (). [Lutz] B.Giebel and Orally Active Cytokines:Designing a Small Molecule Mimetic of Erythropoietin, IBC Conference: New Advances in Peptidemimetics & Small Molecule Design, March 23-25, 1994. In such a case, in order to present application in a clinical field with possible recombinant or low molecular weight compound, it is required to screen such erythropoietin activity simply and certainly. If it is the increased cell which depends for on erythropoietin specifically, it can be used for such a purpose.
[0004] Establishment of the human cell stock increased depending on erythropoietin is reported (Kitamura et al., J.Cell.Physiol.140:323, 1989). However, it is not specifically dependent only on erythropoietin and the cell strain named this TF-1 is a dependency also GM-CSF (granulocyte macrophage colony-stimulating factor) and IL-3 (interleukin 3).
[0005] Moreover, it is a dependency GM-CSF and IL-3 as well as [Homo sapiens megakaryoblastic leukemia cell strain UT-7 (Komatsu et al., Cancer Res.51:341, 1991) which can be maintained under existence of erythropoietin] TF-1 cell. On the other hand, establishment of the UT-7/Epo cell which disappeared GM-CSF and IL-3 dependency is reported (Komatsu et al., Blood 82:456, 1993). However, if GM-CSF of a UT-7/Epo cell and disappearance of IL-3 dependency are the things resulting from quantitative reduction of the acceptor beta chain which are GM-CSF and signal transfer protein common to IL-3 at all (Komatsu et al., above), possibility of having the SCF dependency (Auffray et al., Exp.Hematol.22:417, 1994) of UT-7 cell which is an old stock cannot be denied.
[0006] moreover, recent years and erythropoietin -- the elucidation of a specific signal transfer device is performed energetically, and many matter in connection with it is identified. However,

these researches are done using the cell (for example, Showers et al., Blood 80:3070, 1992) which made TF-1 cell (Hanazono et al., Int.J.Hematol.59:80, 1994) mentioned above and Ba/F3 cell which is a B cell system discover an erythropoietin acceptor artificially, and these cells show a dependency to not only erythropoietin but GM-CSF and IL-3. When the cell reacted to such various cytokine is used, even if it is the case where it stimulates by erythropoietin, possibility of having diverted the signal transfer path of other cytokine cannot be denied.

[0007] Therefore, it was established under erythropoietin existence, and even if it piled up the passage, the cell strain which increases only depending on erythropoietin and can be used also for discovery of signal transfer protein / gene specific to erythropoietin was not obtained.

[0008]

[Problem(s) to be Solved by the Invention] Establishment of the cell strain which can leave the property of the erythroblast which is a physiological erythropoietin target cell, and can depend for only on erythropoietin specifically, can increase in such a situation, and can use for screening of erythropoietin activity, and can be used also for discovery of signal transfer protein / gene specific to erythropoietin is called for.

[0009] The purpose of this invention is to offer the erythropoietin dependency cell strain which can be used for screening of erythropoietin activity.

[0010] This invention also makes it the purpose to offer further the in vitro screening procedure which measures erythropoietin activity using this cell strain.

[0011]

[Means for Solving the Problem] As a result of inquiring wholeheartedly that the above-mentioned purpose should be attained, out of the cell which the erythroblast which is the physiological target cell of erythropoietin leukemia-ized, this invention persons succeeded in establishing the starting cell strain, and completed this invention.

[0012] That is, this invention offers the increased human cell stock which depends for on erythropoietin specifically.

[0013] Furthermore, the in vitro screening procedure of the erythropoietin activity which consists of adding the compound guessed that this invention has erythropoietin activity to the above-mentioned cell strain, and examining cell proliferation or differentiation inducing is offered.

[0014] The cell strain of this invention can be established using an extraction **** cell from for example, an initial erythroblast leukemia (early erhthroblastic leukemia) patient's bone marrow etc. Especially in this invention, the myeloblast of the patient by whom the blast cell was accepted in bone marrow 76.2% was extracted. This cell formed the colony by Epo addition. When this blast cell was furthermore cultivated in FCS (fetal calf serum) and IMDM (chair cove alteration Dulbecco culture medium) which carried out Epo addition, the passage was able to become possible, and the stable cell strain was able to be established. Thus, it is named AS-E2 and the established cell strain is deposited with the National Institute of Bioscience and Human-Technology patent microorganism deposition pin center,large by trust number FIRM:BP -4819 (October 5, 1994 deposition).

[0015] The par pxodase reaction (PO) is negative and AS-E2 accepted megaloblast Mr. change (periodic acid-Schiff reagent-AS positivity).

[0016] Moreover, when the surface antigen of cell strain AS-E2 was examined, CD71, CD36, glycophorins A (GPA), CD38, and CD19, etc. were discovered.

[0017] When the reactivity over the various cytokine of AS-E2 was examined, only when it incubated with Epo, it was stimulated notably, and it was shown that AS-E2 reacts to Epo specifically, and increases. Furthermore, in order to examine the growth inhibition by the reactivity of Epo to AS-E2, and the anti-Epo antibody, when the number of cells was counted under the microscope and the viable count was counted by the trypan blue, it became clear that cell proliferation remarkable only in Epo addition conditions is accepted, and such growth effectiveness is controlled by addition of an anti-Epo antibody.

[0018] Moreover, the manifestation of the Epo acceptor in AS-E2 was checked from the joint

experiment of Radiolabel Epo. As a result of analyzing the property of an Epo acceptor using Scatchard analysis from the data furthermore obtained, the Epo acceptor which AS-E2 discovers was only a low compatibility mold.

[0019] Recently, Epo combines with an Epo acceptor, phosphorylation of Jak2 which is one sort of Jak family tyrosine kinase is carried out by this, and the transfer path of the growth signal that Jak2 self phosphorizes the following molecule with kinase activity is further advocated by this phosphorylation (Ihle et al., TIBS 19:222, 1994). In order to examine the justification of this assumption, after cultivating AS-E2 under Epo nonexistence for 24 hours and adding Epo subsequently, the cell was solubilized and immunoprecipitation was performed by anti-Jak2 antibody or the anti-Epo acceptor antibody. After giving sediment to SDS-PAGE, as a result of imprinting on the PVDF film and detecting thyrosin phosphorylation protein by the anti-phosphorylated tyrosine antibody, AS-E2 reacted to Epo, and the Epo acceptor and the thyrosin phosphorylation of Jak2 which are an Epo signal transfer system were accepted. This can call it the result to suggest that the above-mentioned growth signal transfer path is right.

[0020] the erythrocyte sent from the same acceptor apart from a growth signal -- although existence of the signal of specific cell differentiation is suggested -- this -- current -- it is not solved most. however, the erythrocyte from an Epo acceptor -- the erythrocyte of the lower stream of a river which the signal which activates a series of specific transcription factor groups is transmitted, and is controlled by these transcription factors -- the imprint of a specific gene cluster (a heme synthetase gene and globin gene) is activated, and it is thought that it finally specializes in a hemoglobin production cell (Yamamoto M. the experimental medicine 11:1026 and 1993). the place which examined the manifestation of mRNA of GATA-1 and GATA-2 which is this specific. transcription factor by the Northern blot -- AS-E2 -- both GATA-1 and GATA-2 -- although -- it was checked that it is discovered.

[0021] It was shown that it is the cell strain for which AS-E2 depends only on Epo specifically and which it increases from the various descriptions of such AS-E2.

[0022] Therefore, the cell strain of this invention is useful although Epo activity, such as a low molecular weight compound imagined to have the various recombinant Epo or Epo activity, is screened by in vitro one simply and certainly. Such screening can be carried out by examining cell proliferation or differentiation inducing. For example, the incubation of the compound imagined to have Epo activity and the cell strain of this invention is mixed and carried out. The cell proliferation after fixed time amount The incorporation trial of 3T-thymidine, Or [whether it measures by the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay method (J.Immun.Methods 65:55, 1983 reference), and] Or it can carry out by measuring a viable count etc. Moreover, it is also possible to measure Epo concentration by this examining method. Furthermore, the cell strain of this invention can be used as the symptoms analysis and the therapy model of initial erythroblast leukemia. Furthermore, as mentioned above, the cell strain of this invention can be used also for the experiment for solving specific signal transfer protein / gene to erythropoietin.

[0023] Although an example explains this invention in more detail below, the range of this invention is not limited to this.

[0024]

[Example]

Example 1: Bone marrow liquid was extracted from establishment of AS-E2, and the initial erythroblast leukemia patient of maintenance. As for this patient, the blast cell [light microscope PO (-) PAS(-) CD11b(-) CD13(+) CD33(+) CD10(-) CD19(-) glycophorin A(+) chromosome:46, XY] was accepted in bone marrow 76.2% at the time of the first medical examination. This patient's myeloblast formed the colony by Epo addition. Subsequently, it is this blast cell Epo (2u/ml) and 20% It was stabilized when cultivated in the FCS addition IMDM, and the passage became possible, and it was established as a cell strain. This cell strain was named AS-E2.

[0025] The gestalt and surface antigen of AS-E2 are as follows.

When gestalt AS-E2 of AS-E2 is observed under a microscope, it is about 30micro of ****, and has the vacuole. PO was negative and PAS was a positivity.
The fluorescent antibody technique (Fried et al., Flow Cytometry, Boca Raton, CRC Press:59-78, 1989) by flow cytometer considered the manifestation of the surface antigen of cell strain AS-E2 established by analysis this example of the surface antigen of AS-E2 using the panel of the monoclonal antibody to various Homo sapiens antigens.
[0026] The obtained result is shown in the following table 1.
[0027]
table 1 [ ] The rate of a positivity (%) CD2 0.2 CD10 0.2 CD19 6.5 CD25 0 CD34 0.7 CD36 98.8 CD38 14.1 CD41 0.7CDs71 99.1 HLA-DR 1.1 glycophorin A AS-E2 had discovered CD71, CD36, glycophorins A (GPA), CD38, and CD19, etc. so that clearly from 43.1 tables.
[0028] Example 2: The reactivity to the various cytokine of cell strain AS-E2 established in the reactant example 1 over various cytokine was examined.
[0029] AS-E2 Various cytokine is added to 5,000 cells / well, and it is 5%. CO2, 95% It cultivated for three days at air (100% of humidity), and 37 degrees C. The class of used cytokine The following passes along concentration and it comes out. And a certain :human erythropoietin : 10u/ml Homo sapiens interleukin 1 alpha (hEPO) :10 ng/ml Homo sapiens interleukin 1 beta (hIL-1alpha) :10 ng/ml Homo sapiens interleukin 2 (hIL-1beta) :10 ng/ml Homo sapiens interleukin 3 (hIL-2) :100 ng/ml Homo sapiens interleukin 4 (hIL-3) :10 ng/ml Homo sapiens interleukin 6 (hIL-4) :10 ng/ml Homo sapiens interleukin 7 (hIL-6) :10 ng/ml Homo sapiens interleukin 8 (hIL-7) (hIL-8) :1000 ng/ml Homo sapiens 11 (hIL-11):10 ng/ml interleukin Homo sapiens granulocyte colony-stimulating factor (hG-CSF): -- 100u/ml Homo sapiens granulocyte macrophage colony-stimulating factor (hGM-CSF): -- 10 ng/ml The Homo sapiens leukemia inhibitor : 10 ng/ml human erythrocyte differentiator (hLIF) : 10 ng/ml Homo sapiens stem cell factor (hEDF) : (hSCF) 10 ng(s)/ml Homo sapiens transforming growth factor - beta : (HTGF-beta) 100 ng(s)/ml Homo sapiens basicity fibroblast growth factor (hbFGF) :10 ng/ml Homo sapiens hepatocyte growth factor (hHGF): -- 500 ng/ml Homo sapiens M:100 ng/ml oncostatin Homo sapiens epidermal growth factor (hEGF): -- after 1000 ng/ml culture -- CellTiter96TM AQueous Non-Radioactive Cell Proriferation Assay The number of cells was measured by measuring the absorbance in 490nm using Kit (product made from Promega). The blank which does not contain a cell, and the contrast which does not contain cytokine were examined to coincidence. The obtained result is shown in drawing 1 . Only when it incubated with Epo, growth was stimulated notably, and AS-E2 was not stimulated depending on other cytokine so that clearly from drawing.
Example 3: The Epo reactivity (growth activity) of cell strain AS-E2 established in the growth inhibition example 1 by Epo reactivity and the anti-Epo antibody and the growth activity by the anti-Epo antibody were examined.
[0030] without it adds (1) Epo using 1xAS-E2 cell 105 cells / ml -- culture and (2) Epo 2u/ml -- adding -- culture and (3) Epo 2u/ml and an anti-Epo antibody (it is diluted and used for alphaEpo:1/100) -- adding -- cultivating -- being with time (2, 4, 6, and 8 days) -- the number of cells was counted under the microscope. The obtained result is shown in drawing 2 . As compared with the cell in which the cell which carried out Epo addition does not carry out Epo addition, the remarkable increment in the number of cells was observed so that clearly from drawing. Moreover, when an anti-Epo antibody was added to coincidence, this growth activity was checked.
[0031] Subsequently, the result of having dyed the cell of the three above-mentioned group by the trypan blue, and having counted the viable count is shown in drawing 3 . Although the viable count was decreasing with time in the cell which does not carry out Epo addition, and the cell which added Epo and an anti-Epo antibody so that clearly from drawing, most which was counted as the number of cells counted also as a viable count in the cell which carried out Epo addition.
Example 4: It checked by the following approaches that the Epo acceptor was discovered on the check AS-E2 cell of a manifestation of an Epo acceptor.

[0032] AS-E2 cell was cultivated under 24-hour Epo nonexistence, 0.06-3nM addition of 125 I-Epo (product made from Amersham) was carried out to 1x106 cells, and joint assay was performed on 15 degrees C and the conditions of 3 hours. Nonspecific association was measured under the non-indicator Epo existence of the amount of 400 times of 125 I-Epo. In addition, the presentation of a joint assay culture medium is 2%. BSA, 0.2% It is an IMDM culture medium containing NaN3. Obtained association is shown in drawing 4 . From this, it was checked that the Epo acceptor is discovered on AS-E2 cell.

[0033] Subsequently, when Scatchard analysis was performed from the data obtained from joint assay and having been asked for the dissociation constant (Kd value) and the number of acceptors of an Epo acceptor, it was shown that the Epo acceptor which AS-E2 cell has discovered is one kind of low compatibility mold.

Example 5: The phosphorylation of the Jak2 and the Epo acceptor in Jak2 and phosphorylation AS-E2 of an Epo acceptor was examined by the following approaches, and the signal transfer path of Epo was examined.

[0034] After having cultivated AS-E2 under Epo nonexistence for 24 hours, adding Epo (0 or 10u/ml) subsequently and making it react for 5 minutes, 1% NP-40 (Nakarai Tesuku, Inc. make), 10% Glycerol, 1mM PMSF (the Wako Pure Chem Industries make) and 0.1mg/ml Leupeptin (the Wako Pure Chem Industries make), 5microM pepstatin A (the Wako Pure Chem Industries make), 1mM EDTA, 2mM The cell was solubilized with the tris buffer salting in liquid containing alt.banazin san sodium (SIGMA company make). Immunoprecipitation was performed using this by anti-Jak2 antibody or the anti-Epo acceptor antibody (all are UPSTATE BIOTECHNOLOGY and INC. (USA) company make). After giving sediment to SDS-PAGE, it imprinted on the PVDF film and thyrosin phosphorylation protein was detected by the anti-phosphorylated tyrosine antibody (Wako Pure Chem, Inc. make). the obtained result is shown in drawing 5 (A:anti-Jak2 antibody, a B:anti-Epo acceptor antibody, and A and B -- also in any, a lane 1 expresses the case where a lane 2 carries out Epo addition of the Epo case of not adding). AS-E2 reacted to Epo, and the thyrosin phosphorylation of Jak2 which is an Epo signal transfer system, and an Epo acceptor was accepted so that clearly from drawing.

Example 6: The following Northern blot analysis considered the manifestation of mRNA of GATA-1, GATA-1 in manifestation AS-E2 of mRNA of GATA-2, and GATA-2.

[0035] All RNA was prepared according to the formula of kit attachment from K562 cell strain (chronic-myelogenous-leukemia acute transformation cell strain) and HL-60 cell strain (acute leukemia cell strain), using ISOGEN (NIPPON GENE make) as cell strain AS-E2 and contrast of this invention. Subsequently, Northern blot analysis (Molecular Cloning: A Laboratory Manual, Sambrook et al., Cold Spring Harbor Laboratory Press, 1989) was performed. The indicator of a probe is Random. Primer It carried out using LabellingKit (product made from Takara). Namely, Eco of a PVC vector GATA-1 (Zon et al.; Proc.Natl.Acad.Sci.USA 88:10638-10641, 1991) inserted in RI part and the insertion which codes GATA-2 (Nagai et al., Blood 84:1074-1084, 1994) were started, and the indicator probe was produced according to the formula of kit attachment.

[0036] After doing 30microg use per one lane of all RNA from each above-mentioned cell and performing agarose gel electrophoresis, Hybond-N (product made from Amersham) was used as a hybridization transfer membrane, and blotting was carried out by the capillary tube method. Hybridization is 50%. A formamide, 5xSSPE, 2xDenhardt's solution, 0.1% It carried out at 42 degrees C overnight using the solution which consists of a presentation of SDS. They are after hybridization and a membrane 2xSSC and 0.1% The room temperature washed 4 times by SDS, autoradiography was further performed in BAS (product made from Fuji), and image analysis was carried out. the result is shown in drawing 6 (A:GATA -1, B:GATA -2, and A and B -- all are boiled and, in AS-E2, a lane 2K562, and a lane 3, a lane 1 expresses HL-60). GATA-1 was detected by AS-E 2 andK562 as a clear band, and GATA-2 were accepted by any cell strain of AS-E2, K562, and HL-60 so that clearly from drawing.

[0037]
[Effect of the Invention] By this invention, it was specifically dependent only on erythropoietin, and increased, and the establishment of a cell strain which can be used for screening of erythropoietin activity was attained. The in vitro screening procedure which measures the erythropoietin activity of the compound presumed to have erythropoietin activity using the cell strain of this invention became possible. This in vitro screening can be carried out by examining the cell proliferation or differentiation inducing of a cell strain of this invention. Moreover, it is also possible to measure Epo concentration by this examining method. Furthermore, the cell strain of this invention can be used as the symptoms analysis and the therapy model of initial erythroblast leukemia. Furthermore, the experiment for solving specific signal transfer protein / gene to erythropoietin can be conducted using the cell strain of this invention.
[0038] The utility value of the erythropoietin dependency human cell stock of these points to this invention is very large.

[Translation done.]

* NOTICES *

1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

TECHNICAL FIELD

[Industrial Application] This invention relates to the screening procedure of erythropoietin activity using the increased human cell stock which depends for specifically [it is still more detailed and] to erythropoietin, and this cell strain about an erythropoietin dependency human cell stock.

[Translation done.]

* NOTICES *

1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

PRIOR ART

[Description of the Prior Art] Erythropoietin (it may be hereafter written as Epo) is a glycoprotein which controls cell proliferation differentiation of the erythrocyte in a higher animal (for example, Carnot et al., Compt.Rend.143:384, 1986 reference). Although an erythrocyte is produced by maturation and differentiation of erythroblast in bone marrow, erythropoietin is cytokine which acts on an undifferentiated precursor cell and guides the differentiation to the erythrocyte. From such an operation, erythropoietin is greatly used as a clinical treatment medicine of ischemia, especially renal anemia in recent years.

[0003] Most erythropoietin currently used in the current clinical field is the various recombinant obtained by gene modification technology. Moreover, in recently, it is not recombination protein itself [such]. With low-molecular, more erythropoietin activity To replace with the compound which it has is tried (). [Lutz] B.Giebel and Orally Active Cytokines:Designing a Small Molecule Mimetic of Erythropoietin, IBC Conference: New Advances in Peptidemimetics & Small Molecule Design, March 23-25, 1994. In such a case, in order to present application in a clinical field with possible recombinant or low molecular weight compound, it is required to screen such erythropoietin activity simply and certainly. If it is the increased cell which depends for on erythropoietin specifically, it can be used for such a purpose.

[0004] Establishment of the human cell stock increased depending on erythropoietin is reported (Kitamura et al., J.Cell.Physiol.140:323, 1989). However, it is not specifically dependent only on erythropoietin and the cell strain named this TF-1 is a dependency also GM-CSF (granulocyte macrophage colony-stimulating factor) and IL-3 (interleukin 3).

[0005] Moreover, it is a dependency GM-CSF and IL-3 as well as [Homo sapiens megakaryoblastic leukemia cell strain UT-7 (Komatsu et al., Cancer Res.51:341, 1991) which can be maintained under existence of erythropoietin] TF-1 cell. On the other hand, establishment of the UT-7/Epo cell which disappeared GM-CSF and IL-3 dependency is reported (Komatsu et al., Blood 82:456, 1993). However, if GM-CSF of a UT-7/Epo cell and disappearance of IL-3 dependency are the things resulting from quantitative reduction of the acceptor beta chain which are GM-CSF and signal transfer protein common to IL-3 at all (Komatsu et al., above), possibility of having the SCF dependency (Auffray et al., Exp.Hematol.22:417, 1994) of UT-7 cell which is an old stock cannot be denied.

[0006] moreover, recent years and erythropoietin -- the elucidation of a specific signal transfer device is performed energetically, and many matter in connection with it is identified. However, these researches are done using the cell (for example, Showers et al., Blood 80:3070, 1992) which made TF-1 cell (Hanazono et al., Int.J.Hematol.59:80, 1994) mentioned above and Ba/F3 cell which is a B cell system discover an erythropoietin acceptor artificially, and these cells show a dependency to not only erythropoietin but GM-CSF and IL-3. When the cell reacted to such various cytokine is used, even if it is the case where it stimulates by erythropoietin, possibility of having diverted the signal transfer path of other cytokine cannot be denied.

[0007] Therefore, it was established under erythropoietin existence, and even if it piled up the passage, the cell strain which increases only depending on erythropoietin and can be used also for discovery of signal transfer protein / gene specific to erythropoietin was not obtained.

[Translation done.]

* NOTICES *

1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

EFFECT OF THE INVENTION

[Effect of the Invention] By this invention, it was specifically dependent only on erythropoietin, and increased, and the establishment of a cell strain which can be used for screening of erythropoietin activity was attained. The in vitro screening procedure which measures the erythropoietin activity of the compound presumed to have erythropoietin activity using the cell strain of this invention became possible. This in vitro screening can be carried out by examining the cell proliferation or differentiation inducing of a cell strain of this invention. Moreover, it is also possible to measure Epo concentration by this examining method. Furthermore, the cell strain of this invention can be used as the symptoms analysis and the therapy model of initial erythroblast leukemia. Furthermore, the experiment for solving specific signal transfer protein / gene to erythropoietin can be conducted using the cell strain of this invention.
[0038] The utility value of the erythropoietin dependency human cell stock of these points to this invention is very large.

[Translation done.]

* NOTICES *

1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

TECHNICAL PROBLEM

[Problem(s) to be Solved by the Invention] Establishment of the cell strain which can leave the property of the erythroblast which is a physiological erythropoietin target cell, and can depend for only on erythropoietin specifically, can increase in such a situation, and can use for screening of erythropoietin activity, and can be used also for discovery of signal transfer protein / gene specific to erythropoietin is called for.
[0009] The purpose of this invention is to offer the erythropoietin dependency cell strain which can be used for screening of erythropoietin activity.
[0010] This invention also makes it the purpose to offer further the in vitro screening procedure which measures erythropoietin activity using this cell strain.

[Translation done.]

* NOTICES *

1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

MEANS

[Means for Solving the Problem] As a result of inquiring wholeheartedly that the above-mentioned purpose should be attained, out of the cell which the erythroblast which is the physiological target cell of erythropoietin leukemia-ized, this invention persons succeeded in establishing the starting cell strain, and completed this invention.
[0012] That is, this invention offers the increased human cell stock which depends for on erythropoietin specifically.
[0013] Furthermore, the in vitro screening procedure of the erythropoietin activity which consists of adding the compound guessed that this invention has erythropoietin activity to the above-mentioned cell strain, and examining cell proliferation or differentiation inducing is offered.
[0014] The cell strain of this invention can be established using an extraction **** cell from for example, an initial erythroblast leukemia (early erhthroblastic leukemia) patient's bone marrow etc. Especially in this invention, the myeloblast of the patient by whom the blast cell was accepted in bone marrow 76.2% was extracted. This cell formed the colony by Epo addition. When this blast cell was furthermore cultivated in FCS (fetal calf serum) and IMDM (chair cove alteration Dulbecco culture medium) which carried out Epo addition, the passage was able to become possible, and the stable cell strain was able to be established. Thus, it is named AS-E2 and the established cell strain is deposited with the National Institute of Bioscience and Human-Technology patent microorganism deposition pin center,large by trust number FIRM:BP -4819 (October 5, 1994 deposition).
[0015] The par pxodase reaction (PO) is negative and AS-E2 accepted megaloblast Mr. change (periodic acid-Schiff reagent-AS positivity).
[0016] Moreover, when the surface antigen of cell strain AS-E2 was examined, CD71, CD36, glycophorins A (GPA), CD38, and CD19, etc. were discovered.
[0017] When the reactivity over the various cytokine of AS-E2 was examined, only when it incubated with Epo, it was stimulated notably, and it was shown that AS-E2 reacts to Epo specifically, and increases. Furthermore, in order to examine the growth inhibition by the reactivity of Epo to AS-E2, and the anti-Epo antibody, when the number of cells was counted under the microscope and the viable count was counted by the trypan blue, it became clear that cell proliferation remarkable only in Epo addition conditions is accepted, and such growth effectiveness is controlled by addition of an anti-Epo antibody.
[0018] Moreover, the manifestation of the Epo acceptor in AS-E2 was checked from the joint experiment of Radiolabel Epo. As a result of analyzing the property of an Epo acceptor using Scatchard analysis from the data furthermore obtained, the Epo acceptor which AS-E2 discovers was only a low compatibility mold.
[0019] Recently, Epo combines with an Epo acceptor, phosphorylation of Jak2 which is one sort of Jak family tyrosine kinase is carried out by this, and the transfer path of the growth signal that Jak2 self phosphorizes the following molecule with kinase activity is further advocated by this phosphorylation (Ihle et al., TIBS 19:222, 1994). In order to examine the justification of this

assumption, after cultivating AS-E2 under Epo nonexistence for 24 hours and adding Epo subsequently, the cell was solubilized and immunoprecipitation was performed by anti-Jak2 antibody or the anti-Epo acceptor antibody. After giving sediment to SDS-PAGE, as a result of imprinting on the PVDF film and detecting thyrosin phosphorylation protein by the anti-phosphorylated tyrosine antibody, AS-E2 reacted to Epo, and the Epo acceptor and the thyrosin phosphorylation of Jak2 which are an Epo signal transfer system were accepted. This can call it the result to suggest that the above-mentioned growth signal transfer path is right.

[0020] the erythrocyte sent from the same acceptor apart from a growth signal -- although existence of the signal of specific cell differentiation is suggested -- this -- current -- it is not solved most. however, the erythrocyte from an Epo acceptor -- the erythrocyte of the lower stream of a river which the signal which activates a series of specific transcription factor groups is transmitted, and is controlled by these transcription factors -- the imprint of a specific gene cluster (a heme synthetase gene and globin gene) is activated, and it is thought that it finally specializes in a hemoglobin production cell (Yamamoto M. the experimental medicine 11:1026 and 1993). the place which examined the manifestation of mRNA of GATA-1 and GATA-2 which is this specific transcription factor by the Northern blot -- AS-E2 -- both GATA-1 and GATA-2 -- although -- it was checked that it is discovered.

[0021] It was shown that it is the cell strain for which AS-E2 depends only on Epo specifically and which it increases from the various descriptions of such AS-E2.

[0022] Therefore, the cell strain of this invention is useful although Epo activity, such as a low molecular weight compound imagined to have the various recombinant Epo or Epo activity, is screened by in vitro one simply and certainly. Such screening can be carried out by examining cell proliferation or differentiation inducing. For example, the incubation of the compound imagined to have Epo activity and the cell strain of this invention is mixed and carried out. The cell proliferation after fixed time amount The incorporation trial of 3T-thymidine, Or [whether it measures by the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay method (J.Immun.Methods 65:55, 1983 reference), and] Or it can carry out by measuring a viable count etc. Moreover, it is also possible to measure Epo concentration by this examining method. Furthermore, the cell strain of this invention can be used as the symptoms analysis and the therapy model of initial erythroblast leukemia. Furthermore, as mentioned above, the cell strain of this invention can be used also for the experiment for solving specific signal transfer protein / gene to erythropoietin.

[0023] Although an example explains this invention in more detail below, the range of this invention is not limited to this.

[Translation done.]

* NOTICES *

1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

EXAMPLE

[Example]
Example 1: Bone marrow liquid was extracted from establishment of AS-E2, and the initial erythroblast leukemia patient of maintenance. As for this patient, the blast cell [light microscope PO (-) PAS(-) CD11b(-) CD13(+) CD33(+) CD10(-) CD19(-) glycophorin A(+) chromosome:46, XY] was accepted in bone marrow 76.2% at the time of the first medical examination. This patient's myeloblast formed the colony by Epo addition. Subsequently, it is this blast cell Epo (2u/ml) and 20% It was stabilized when cultivated in the FCS addition IMDM, and the passage became possible, and it was established as a cell strain. This cell strain was named AS-E2.
[0025] The gestalt and surface antigen of AS-E2 are as follows.
When gestalt AS-E2 of AS-E2 is observed under a microscope, it is about 30micro of ****, and has the vacuole. PO was negative and PAS was a positivity.
The fluorescent antibody technique (Fried et al., Flow Cytometry, Boca Raton, CRC Press:59-78, 1989) by flow cytometer considered the manifestation of the surface antigen of cell strain AS-E2 established by analysis this example of the surface antigen of AS-E2 using the panel of the monoclonal antibody to various Homo sapiens antigens.
[0026] The obtained result is shown in the following table 1.
[0027]
table 1 [ ] The rate of a positivity (%) CD2 0.2 CD10 0.2 CD19 6.5 CD25 0 CD34 0.7 CD36 98.8 CD38 14.1 CD41 0.7CDs71 99.1 HLA-DR 1.1 glycophorin A AS-E2 had discovered CD71, CD36, glycophorins A (GPA), CD38, and CD19, etc. so that clearly from 43.1 tables.
[0028] Example 2: The reactivity to the various cytokine of cell strain AS-E2 established in the reactant example 1 over various cytokine was examined.
[0029] AS-E2 Various cytokine is added to 5,000 cells / well, and it is 5%. CO2, 95% It cultivated for three days at air (100% of humidity), and 37 degrees C. The class of used cytokine The following passes along concentration and it comes out. And a certain :human erythropoietin : 10u/ml Homo sapiens interleukin 1 alpha (hEPO) :10 ng/ml Homo sapiens interleukin 1 beta (hIL-1alpha) :10 ng/ml Homo sapiens interleukin 2 (hIL-1beta) :10 ng/ml Homo sapiens interleukin 3 (hIL-2) :100 ng/ml Homo sapiens interleukin 4 (hIL-3) :10 ng/ml Homo sapiens interleukin 6 (hIL-4) :10 ng/ml Homo sapiens interleukin 7 (hIL-6) :10 ng/ml Homo sapiens interleukin 8 (hIL-7) (hIL-8) :1000 ng/ml Homo sapiens 11 (hIL-11):10 ng/ml interleukin Homo sapiens granulocyte colony-stimulating factor (hG-CSF): -- 100u/ml Homo sapiens granulocyte macrophage colony-stimulating factor (hGM-CSF): -- 10 ng/ml The Homo sapiens leukemia inhibitor : 10 ng/ml human erythrocyte differentiator (hLIF) : 10 ng/ml Homo sapiens stem cell factor (hEDF) : (hSCF) 10 ng(s)/ml Homo sapiens transforming growth factor - beta : (HTGF-beta) 100 ng(s)/ml Homo sapiens basicity fibroblast growth factor (hbFGF) :10 ng/ml Homo sapiens hepatocyte growth factor (hHGF): -- 500 ng/ml Homo sapiens M:100 ng/ml oncostatin Homo sapiens epidermal growth factor (hEGF): -- after 1000 ng/ml culture -- CellTiter96TM AQueous Non-Radioactive Cell Proriferation Assay The number of cells was

measured by measuring the absorbance in 490nm using Kit (product made from Promega). The blank which does not contain a cell, and the contrast which does not contain cytokine were examined to coincidence. The obtained result is shown in drawing 1 . Only when it incubated with Epo, growth was stimulated notably, and AS-E2 was not stimulated depending on other cytokine so that clearly from drawing.

Example 3: The Epo reactivity (growth activity) of cell strain AS-E2 established in the growth inhibition example 1 by Epo reactivity and the anti-Epo antibody and the growth activity by the anti-Epo antibody were examined.

[0030] without it adds (1) Epo using 1xAS-E2 cell 105 cells / ml -- culture and (2) Epo 2u/ml -- adding -- culture and (3) Epo 2u/ml and an anti-Epo antibody (it is diluted and used for alphaEpo:1/100) -- adding -- cultivating -- being with time (2, 4, 6, and 8 days) -- the number of cells was counted under the microscope. The obtained result is shown in drawing 2 . As compared with the cell in which the cell which carried out Epo addition does not carry out Epo addition, the remarkable increment in the number of cells was observed so that clearly from drawing. Moreover, when an anti-Epo antibody was added to coincidence, this growth activity was checked.

[0031] Subsequently, the result of having dyed the cell of the three above-mentioned group by the trypan blue, and having counted the viable count is shown in drawing 3 . Although the viable count was decreasing with time in the cell which does not carry out Epo addition, and the cell which added Epo and an anti-Epo antibody so that clearly from drawing, most which was counted as the number of cells counted also as a viable count in the cell which carried out Epo addition.

Example 4: It checked by the following approaches that the Epo acceptor was discovered on the check AS-E2 cell of a manifestation of an Epo acceptor.

[0032] AS-E2 cell was cultivated under 24-hour Epo nonexistence, 0.06-3nM addition of 125 I-Epo (product made from Amersham) was carried out to 1x106 cells, and joint assay was performed on 15 degrees C and the conditions of 3 hours. Nonspecific association was measured under the non-indicator Epo existence of the amount of 400 times of 125 I-Epo. In addition, the presentation of a joint assay culture medium is 2%. BSA, 0.2% It is an IMDM culture medium containing NaN3. Obtained association is shown in drawing 4 . From this, it was checked that the Epo acceptor is discovered on AS-E2 cell.

[0033] Subsequently, when Scatchard analysis was performed from the data obtained from joint assay and having been asked for the dissociation constant (Kd value) and the number of acceptors of an Epo acceptor, it was shown that the Epo acceptor which AS-E2 cell has discovered is one kind of low compatibility mold.

Example 5: The phosphorylation of the Jak2 and the Epo acceptor in Jak2 and phosphorylation AS-E2 of an Epo acceptor was examined by the following approaches, and the signal transfer path of Epo was examined.

[0034] After having cultivated AS-E2 under Epo nonexistence for 24 hours, adding Epo (0 or 10u/ml) subsequently and making it react for 5 minutes, 1% NP-40 (Nakarai Tesuku, Inc. make), 10% Glycerol, 1mM PMSF (the Wako Pure Chem Industries make) and 0.1mg/ml Leupeptin (the Wako Pure Chem Industries make), 5microM pepstatin A (the Wako Pure Chem Industries make), 1mM EDTA, 2mM The cell was solubilized with the tris buffer salting in liquid containing alt.banazin san sodium (SIGMA company make). Immunoprecipitation was performed using this by anti-Jak2 antibody or the anti-Epo acceptor antibody (all are UPSTATE BIOTECHNOLOGY and INC. (USA) company make). After giving sediment to SDS-PAGE, it imprinted on the PVDF film and thyrosin phosphorylation protein was detected by the anti-phosphorylated tyrosine antibody (Wako Pure Chem, Inc. make). the obtained result is shown in drawing 5 (A:anti-Jak2 antibody, a B:anti-Epo acceptor antibody, and A and B -- also in any, a lane 1 expresses the case where a lane 2 carries out Epo addition of the Epo case of not adding). AS-E2 reacted to Epo, and the thyrosin phosphorylation of Jak2 which is an Epo signal transfer system, and an Epo acceptor was accepted so that clearly from drawing.

Example 6: The following Northern blot analysis considered the manifestation of mRNA of GATA-1, GATA-1 in manifestation AS-E2 of mRNA of GATA-2, and GATA-2.
[0035] All RNA was prepared according to the formula of kit attachment from K562 cell strain (chronic-myelogenous-leukemia acute transformation cell strain) and HL-60 cell strain (acute leukemia cell strain), using ISOGEN (NIPPON GENE make) as cell strain AS-E2 and contrast of this invention. Subsequently, Northern blot analysis (Molecular Cloning: A Laboratory Manual, Sambrook et al., Cold Spring Harbor Laboratory Press, 1989) was performed. The indicator of a probe is Random. Primer It carried out using LabellingKit (product made from Takara). Namely, Eco of a PVC vector GATA-1 (Zon et al., Proc.Natl.Acad.Sci.USA 88:10638-10641, 1991) inserted in RI part and the insertion which codes GATA-2 (Nagai et al., Blood 84:1074-1084, 1994) were started, and the indicator probe was produced according to the formula of kit attachment.
[0036] After doing 30microg use per one lane of all RNA from each above-mentioned cell and performing agarose gel electrophoresis, Hybond-N (product made from Amersham) was used as a hybridization transfer membrane, and blotting was carried out by the capillary tube method. Hybridization is 50%. A formamide, 5xSSPE, 2xDenhardt's solution, 0.1% It carried out at 42 degrees C overnight using the solution which consists of a presentation of SDS. They are after hybridization and a membrane 2xSSC and 0.1% The room temperature washed 4 times by SDS, autoradiography was further performed in BAS (product made from Fuji), and image analysis was carried out. the result is shown in drawing 6 (A:GATA -1, B:GATA -2, and A and B -- all are boiled and, in AS-E2, a lane 2K562, and a lane 3, a lane 1 expresses HL-60). GATA-1 was detected by AS-E 2 andK562 as a clear band, and GATA-2 were accepted by any cell strain of AS-E2, K562, and HL-60 so that clearly from drawing.

[Translation done.]

* NOTICES *

DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]
[Drawing 1] It is drawing showing the reactivity of the various cytokine to AS-E2 cell.
[Drawing 2] It is drawing showing the number of cells with time observed under the microscope to show the Epo reactivity of AS-E2, and the growth activity by the anti-Epo antibody. (-) is Epo. For the cell which added 2u/ml, and (**), the cell which does not add Epo, and (**) are Epo. The cell which added 2u/ml and an anti-Epo antibody (alphaEpo:1/100 dilution) is expressed.
[Drawing 3] It is drawing showing the viable count with time which carried out trypan blue dyeing to show the Epo reactivity of AS-E2, and the growth activity by the anti-Epo antibody. (-) is Epo. The cell which added 2u/ml, the cell into which (**) does not add Epo, and (**) express the cell which added Epo2u/ml and an anti-Epo antibody (alphaEpo:1/100 dilution).
[Drawing 4] It is drawing showing the joint assay of AS-E2 using 125 I-Epo. In (O), all avidity and (**) express nonspecific avidity, and (-) expresses specific binding activity.
[Drawing 5] It is drawing (photograph of electrophoresis) showing the phosphorylation of the Jak2 and the Epo acceptor in AS-E2. A gives an immune precipitate with an anti-Epo acceptor antibody (lane 1 Epo un-adding; the lane 2 Epo addition) to anti-Jak2 antibody (lane 1 Epo un-adding; the lane 2 Epo addition) among drawing, B is given to the electrophoresis of SDS-PAGE, it imprints on the PVDF film, and drawing which detected thyrosin phosphorylation protein by the anti-phosphorylated tyrosine antibody is expressed.
[Drawing 6] It is drawing (photograph of electrophoresis) showing the manifestation of mRNA of AS-E2, K562, GATA-1 in HL-60 cell strain, and GATA-2. The result of the Northern blot for which A used GATA-1 (lane 1 AS-E2; lane 2K562; the lane 3 HL- 60) for among drawing, and B used GATA-2 (lane 1 AS-E2; lane 2K562; the lane 3 HL- 60) as a probe is expressed.

[Translation done.]

(19)日本国特許庁（ＪＰ）　(12)公開特許公報（Ａ）　(11)特許出願公開番号

特開平8－163982

(43)公開日　平成８年(1996)６月25日

(51)Int.Cl.[6]	識別記号	庁内整理番号	ＦＩ		技術表示箇所
Ｃ１２Ｎ　5/06					
Ａ６１Ｋ　38/00	ＡＢＹ				
	ＡＣＣ				
		9281－4Ｂ	Ｃ１２Ｎ　5/00	Ｅ	
			Ａ６１Ｋ　37/02	ＡＢＹ	

審査請求　未請求　請求項の数４　ＦＤ　(全 10 頁)　最終頁に続く

(21)出願番号　特願平6－298813

(22)出願日　平成６年(1994)11月８日

(31)優先権主張番号　特願平6－272823

(32)優先日　平６(1994)10月12日

(33)優先権主張国　日本（ＪＰ）

(71)出願人　000003311
中外製薬株式会社
東京都北区浮間５丁目５番１号

(72)発明者　朝長　万左男
長崎県長崎市筑後町５－７

(72)発明者　栗山　一孝
長崎県長崎市大手２丁目５番６号

(72)発明者　宮崎　泰司
長崎県長崎市彦見町２－25

(72)発明者　樋口　正人
静岡県御殿場市駒門１丁目135番地　中外製薬株式会社内

(74)代理人　弁理士　湯浅　恭三　(外６名)

(54)【発明の名称】　エリスロポエチン依存性ヒト細胞株

(57)【要約】

【目的】　エリスロポエチンのみに特異的に依存して増殖し、エリスロポエチン活性のスクリーニングに用い得る細胞株を樹立する。

【構成】　エリスロポエチンに特異的に依存して増殖するヒト細胞株、およびエリスロポエチン活性を有すると推察される化合物を上記細胞株に添加して細胞増殖または分化誘導を試験することからなるエリスロポエチン活性のインビトロスクリーニング法。

1

【特許請求の範囲】

【請求項１】　エリスロポエチンに特異的に依存して増殖するヒト細胞株。

【請求項２】　初期赤芽球白血病患者の骨髄細胞由来である請求項１に記載の細胞株。

【請求項３】　ＡＳ－Ｅ２（生命工学工業技術研究所特許微生物寄託センター、受託番号ＦＩＲＭ：ＢＰ－４８１９）である請求項１に記載の細胞株。

【請求項４】　エリスロポエチン活性を有すると推察される化合物を請求項１に記載の細胞株に添加して細胞増殖または分化誘導を試験することからなるエリスロポエチン活性のインビトロスクリーニング法。

【発明の詳細な説明】

【０００１】

【産業上の利用分野】本発明はエリスロポエチン依存性ヒト細胞株に関し、さらに詳しくはエリスロポエチンに特異的に依存して増殖するヒト細胞株、および該細胞株を用いるエリスロポエチン活性のスクリーニング法に関する。

【０００２】

【従来の技術】エリスロポエチン（以下、Ｅｐｏと略記することもある）は高等動物における赤血球の細胞増殖分化を制御する糖タンパク質である（例えば、Carnot et al.,Compt. Rend. 143:384, 1986参照）。赤血球は骨髄中で赤芽球の成熟および分化により生産されるが、エリスロポエチンは未分化の前駆細胞に作用してその赤血球への分化を誘導するサイトカインである。そのような作用から、エリスロポエチンは貧血、特に腎性貧血の臨床治療薬として近年大いに使用されている。

【０００３】現在臨床分野で使用されているエリスロポエチンのほとんどは遺伝子組換え技術によって得られる各種組換え体である。また、最近ではこのような組換えタンパク質そのものではなく、もっと低分子でエリスロポエチン活性をもつ化合物で置き換えることが試みられている（Lutz B. Giebel, Orally Active Cytokines:Designing a Small Molecule Mimetic of Erythropoietin, IBC Conference: New Advances in Peptidemimetics & Small Molecule Design, March 23-25, 1994）。このような場合、可能性のある組換え体または低分子化合物を臨床分野での応用に供するためには、これらのエリスロポエチン活性を簡単かつ確実にスクリーニングすることが必要である。エリスロポエチンに特異的に依存して増殖する細胞であれば、このような目的に使用することができる。

【０００４】エリスロポエチンに依存して増殖するヒト細胞株の樹立が報告されている（Kitamura et al., J. Cell. Physiol. 140:323, 1989）。しかしながら、このＴＦ－１と命名された細胞株はエリスロポエチンのみに特異的に依存するのではなく、ＧＭ－ＣＳＦ（顆粒球マクロファージコロニー刺激因子）やＩＬ－３（インターロイキン－３）にも依存性である。

2

【０００５】また、エリスロポエチンの存在下に維持しうるヒト巨核芽球性白血病細胞株ＵＴ－７（Komatsu et al., Cancer Res. 51:341, 1991）もＴＦ－１細胞と同様にＧＭ－ＣＳＦやＩＬ－３にも依存性である。一方、ＧＭ－ＣＳＦおよびＩＬ－３依存性を消失したＵＴ－７／Ｅｐｏ細胞の樹立が報告されている（Komatsu et al., Blood 82:456, 1993）。しかしながら、ＵＴ－７／Ｅｐｏ細胞のＧＭ－ＣＳＦおよびＩＬ－３依存性の消失が、ＧＭ－ＣＳＦ、ＩＬ－３共通のシグナル伝達タンパク質である受容体β鎖の量的減少に起因しているものである以上（Komatsu et al., 前出）、親株であるＵＴ－７細胞のＳＣＦ依存性（Auffray et al.,Exp. Hematol. 22:417, 1994）を有している可能性は否定できない。

【０００６】また、近年、エリスロポエチン特異的なシグナル伝達機構の解明が精力的に行われ、それにかかわる多くの物質が同定されている。しかし、これらの研究は前述したＴＦ－１細胞（Hanazono et al., Int. J. Hematol. 59:80, 1994）や、Ｂ細胞系であるＢａ／Ｆ３細胞にエリスロポエチン受容体を人為的に発現させた細胞（例えば、Showers et al., Blood 80:3070, 1992）を用いて行われており、これらの細胞はエリスロポエチンのみならず、ＧＭ－ＣＳＦやＩＬ－３にも依存性を示す。このような様々なサイトカインに反応する細胞を用いた場合には、エリスロポエチンで刺激した場合であっても他のサイトカインのシグナル伝達経路を流用している可能性が否定できない。

【０００７】したがって、エリスロポエチン存在下に樹立され、継代を重ねてもエリスロポエチンのみに依存して増殖し、かつエリスロポエチンに特異的なシグナル伝達タンパク質／遺伝子の発見にも使用できるような細胞株は得られていなかった。

【０００８】

【発明が解決すべき課題】このような状況の中で、生理的なエリスロポエチン標的細胞である赤芽球の性質を残し、かつエリスロポエチンのみに特異的に依存して増殖し、エリスロポエチン活性のスクリーニングに用いることができ、またエリスロポエチンに特異的なシグナル伝達タンパク質／遺伝子の発見にも使用できる細胞株の樹立が求められている。

【０００９】本発明の目的はエリスロポエチン活性のスクリーニングに使用できるエリスロポエチン依存性細胞株を提供することにある。

【００１０】本発明はさらに、該細胞株を用いてエリスロポエチン活性を測定するインビトロスクリーニング法を提供することも目的とする。

【００１１】

【課題を解決するための手段】本発明者らは、上記目的を達成すべく鋭意研究した結果、エリスロポエチンの生理的標的細胞である赤芽球が白血病化した細胞の中か

ら、かかる細胞株を樹立することに成功し、本発明を完成した。

【００１２】すなわち、本発明はエリスロポエチンに特異的に依存して増殖するヒト細胞株を提供する。

【００１３】さらに、本発明はエリスロポエチン活性を有すると推察される化合物を上記細胞株に添加して細胞増殖または分化誘導を試験することからなるエリスロポエチン活性のインビトロスクリーニング法を提供する。

【００１４】本発明の細胞株は、例えば初期赤芽球白血病（early erhthroblastic leukemia）患者の骨髄などから採取さた細胞を用いて樹立することができる。本発明では特に、骨髄に芽球が７６．２％認められた患者の骨髄芽球を採取した。この細胞はＥｐｏ添加でコロニーを形成した。さらにこの芽球をＦＣＳ（ウシ胎児血清）およびＥｐｏ添加したＩＭＤＭ（イスコーブ改変ダルベッコ培地）にて培養したところ継代可能となり、安定した細胞株を樹立することができた。このようにして樹立された細胞株はＡＳ－Ｅ２と命名され、生命工学工業技術研究所特許微生物寄託センターに受託番号ＦＩＲＭ：ＢＰ－４８１９で寄託されている（１９９４年１０月５日寄託）。

【００１５】ＡＳ－Ｅ２はパーオキシダーゼ反応（ＰＯ）が陰性であり、巨赤芽球様変化を認めた（過ヨウ素酸シッフ試薬：ＰＡＳ陽性）。

【００１６】また、細胞株ＡＳ－Ｅ２の表面抗原を検討したところ、ＣＤ７１、ＣＤ３６、グリコホリンＡ（ＧＰＡ）、ＣＤ３８、ＣＤ１９などを発現していた。

【００１７】ＡＳ－Ｅ２の各種サイトカインに対する反応性を試験したところ、Ｅｐｏとともにインキュベートしたときのみ顕著に刺激され、ＡＳ－Ｅ２がＥｐｏに特異的に反応して増殖することを示した。さらに、ＡＳ－Ｅ２に対するＥｐｏの反応性および抗Ｅｐｏ抗体による増殖阻害を試験するために顕微鏡下で細胞数を数え、またトリパンブルーで生細胞数をカウントしたところ、Ｅｐｏ添加条件にのみ顕著な細胞増殖が認められ、また抗Ｅｐｏ抗体の添加によってこのような増殖効果は抑制されることが明らかとなった。

【００１８】また、放射標識Ｅｐｏの結合実験よりＡＳ－Ｅ２におけるＥｐｏ受容体の発現が確認された。さらに得られたデータからＳｃａｔｃｈａｒｄ分析を用いてＥｐｏ受容体の性質を解析した結果、ＡＳ－Ｅ２が発現するＥｐｏ受容体は低親和性型のみであった。

【００１９】最近、ＥｐｏはＥｐｏ受容体と結合し、これによってＪａｋファミリーチロシンキナーゼの１種であるＪａｋ２がリン酸化され、さらにこのリン酸化によってＪａｋ２自身がキナーゼ活性をもって次の分子をリン酸化するという増殖シグナルの伝達経路が提唱されている（Ihle et al., TIBS 19:222, 1994）。この仮説の正当性を検討するために、ＡＳ－Ｅ２をＥｐｏ非存在下で２４時間培養し、次いでＥｐｏを添加した後、細胞を可溶化し、抗Ｊａｋ２抗体または抗Ｅｐｏ受容体抗体で免疫沈降を行った。沈降物をＳＤＳ－ＰＡＧＥに付した後、ＰＶＤＦ膜に転写し、抗リン酸化チロシン抗体でチロシンリン酸化タンパク質を検出した結果、ＡＳ－Ｅ２はＥｐｏに反応し、かつＥｐｏシグナル伝達系であるＥｐｏ受容体およびＪａｋ２のチロシンリン酸化が認められた。これは、上記の増殖シグナル伝達経路が正しいことを示唆する結果といえる。

【００２０】増殖シグナルとは別に、同じ受容体から発信される赤血球特異的な細胞分化のシグナルの存在が示唆されているが、これについては現在ほとんど解明されていない。ただ、Ｅｐｏ受容体から赤血球特異的な一連の転写因子群を活性化するシグナルが伝達され、これら転写因子によって制御されている下流の赤血球特異的な遺伝子群（ヘム合成酵素遺伝子やグロビン遺伝子）の転写が活性化され、最終的にヘモグロビン産生細胞に分化すると考えられている（Yamamoto M. 実験医学11:1026, 1993）。この特異的な転写因子であるＧＡＴＡ－１、ＧＡＴＡ－２のｍＲＮＡの発現をノーザンブロットにより試験したところ、ＡＳ－Ｅ２ではＧＡＴＡ－１およびＧＡＴＡ－２のいずれもが発現していることが確認された。

【００２１】このようなＡＳ－Ｅ２の各種特徴から、ＡＳ－Ｅ２がＥｐｏのみに特異的に依存して増殖する細胞株であることが示された。

【００２２】したがって、本発明の細胞株は各種組換え体ＥｐｏまたはＥｐｏ活性を有すると推察される低分子化合物などのＥｐｏ活性を簡単かつ確実にインビトロでスクリーニングするのに有用である。このようなスクリーニングは細胞増殖または分化誘導を試験することによって実施できる。例えば、Ｅｐｏ活性を有すると推察される化合物と本発明の細胞株とを混合してインキュベーションし、一定時間後の細胞増殖を^{3}Ｔ－チミジンの取り込み試験、またはＭＴＴ（3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide）アッセイ法（J. Immun. Methods 65:55, 1983参照）で測定するか、あるいは生細胞数を測定することなどによって実施することができる。また、この試験法によってＥｐｏ濃度を測定することも可能である。さらに、本発明の細胞株を、初期赤芽球白血病の病態解析や治療モデルとして使用することができる。さらには、上述したように、本発明の細胞株を、エリスロポエチンに特異的なシグナル伝達タンパク質／遺伝子を解明するための実験にも使用することができる。

【００２３】以下に本発明を実施例によりさらに詳しく説明するが、本発明の範囲はこれに限定されるものではない。

【００２４】

【実施例】

実施例１：ＡＳ－Ｅ２の樹立および維持

初期赤芽球白血病患者から骨髄液を採取した。この患者は初診時骨髄に芽球［光顕ＰＯ（－）、ＰＡＳ（－）、ＣＤ１１ｂ（－）、ＣＤ１３（＋）、ＣＤ３３（＋）、ＣＤ１０（－）、ＣＤ１９（－）、グリコホリンＡ（＋）、染色体：４６、ＸＹ］が７６．２％認められた。この患者の骨髄芽球はＥｐｏ添加でコロニーを形成した。次いでこの芽球を、Ｅｐｏ（２ｕ／ｍｌ）および２０％　ＦＣＳ添加ＩＭＤＭ中で培養したところ安定して継代可能となり、細胞株として樹立された。この細胞株をＡＳ－Ｅ２と命名した。

【００２５】ＡＳ－Ｅ２の形態および表面抗原は以下の通りである。

ＡＳ－Ｅ２の形態

ＡＳ－Ｅ２は顕微鏡で観察したところ、径約３０μ程度であり、空胞を有している。ＰＯは陰性であり、ＰＡＳは陽性であった。

ＡＳ－Ｅ２の表面抗原の解析

本実施例で樹立した細胞株ＡＳ－Ｅ２の表面抗原の発現を、各種ヒト抗原に対するモノクローナル抗体のパネルを用いて、フローサイトメーターによる蛍光抗体法（Fried et al., Flow Cytometry, Boca Raton, CRC Press: 59-78, 1989）により検討した。

【００２６】得られた結果を以下の表１に示す。

【００２７】

表１

	陽性率（％）
ＣＤ２	０．２
ＣＤ１０	０．２
ＣＤ１９	６．５
ＣＤ２５	０
ＣＤ３４	０．７
ＣＤ３６	９８．８
ＣＤ３８	１４．１
ＣＤ４１	０．７
ＣＤ７１	９９．１
ＨＬＡ－ＤＲ	１．１
グリコホリンＡ	４３．１

表から明らかなように、ＡＳ－Ｅ２はＣＤ７１、ＣＤ３６、グリコホリンＡ（ＧＰＡ）、ＣＤ３８、ＣＤ１９などを発現していた。

【００２８】実施例２：各種サイトカインに対する反応性

実施例１で樹立した細胞株ＡＳ－Ｅ２の各種サイトカインへの反応性を試験した。

【００２９】ＡＳ－Ｅ２　５，０００細胞／ウエルに各種サイトカインを添加し、５％　CO_2、９５％　空気（湿度１００％）、３７℃で３日間培養した。使用したサイトカインの種類および濃度は以下の通りである：

ヒトエリスロポエチン（ｈＥＰＯ）：１０ｕ／ｍｌ
ヒトインターロイキン－１α（ｈＩＬ－１α）：１０ｎｇ／ｍｌ
ヒトインターロイキン－１β（ｈＩＬ－１β）：１０ｎｇ／ｍｌ
ヒトインターロイキン２（ｈＩＬ－２）：１０ｎｇ／ｍｌ
ヒトインターロイキン３（ｈＩＬ－３）：１００ｎｇ／ｍｌ
ヒトインターロイキン４（ｈＩＬ－４）：１０ｎｇ／ｍｌ
ヒトインターロイキン６（ｈＩＬ－６）：１０ｎｇ／ｍｌ
ヒトインターロイキン７（ｈＩＬ－７）：１０ｎｇ／ｍｌ
ヒトインターロイキン８（ｈＩＬ－８）：１０００ｎｇ／ｍｌ
ヒトインターロイキン１１（ｈＩＬ－１１）：１０ｎｇ／ｍｌ
ヒト顆粒球コロニー刺激因子（ｈＧ－ＣＳＦ）：１００ｕ／ｍｌ
ヒト顆粒球マクロファージコロニー刺激因子（ｈＧＭ－ＣＳＦ）：１０ｎｇ／ｍｌ　ヒト白血病阻害因子（ｈＬＩＦ）：１０ｎｇ／ｍｌ
ヒト赤血球分化因子（ｈＥＤＦ）：１０ｎｇ／ｍｌ
ヒト幹細胞因子（ｈＳＣＦ）：１０ｎｇ／ｍｌ
ヒトトランスフォーミング増殖因子－β（ｈＴＧＦ－β）：１００ｎｇ／ｍｌ
ヒト塩基性繊維芽細胞増殖因子（ｈｂＦＧＦ）：１０ｎｇ／ｍｌ
ヒト肝細胞増殖因子（ｈＨＧＦ）：５００ｎｇ／ｍｌ
ヒトオンコスタチンＭ：１００ｎｇ／ｍｌ

ヒト上皮増殖因子（ｈＥＧＦ）：１０００ｎｇ／ｍｌ

培養後にＣｅｌｌＴｉｔｅｒ９６™ ＡＱｕｅｏｕｓ Ｎｏｎ－Ｒａｄｉｏａｃｔｉｖｅ Ｃｅｌｌ Ｐｒｏｒｉｆｅｒａｔｉｏｎ Ａｓｓａｙ Ｋｉｔ（Ｐｒｏｍｅｇａ社製）を用いて４９０ｎｍにおける吸光度を測定することにより細胞数を測定した。細胞を含まないブランク、およびサイトカインを含まない対照を同時に試験した。得られた結果を図１に示す。図から明らかなように、ＡＳ－Ｅ２はＥｐｏとともにインキュベートしたときのみ顕著に増殖が刺激され、その他のサイトカインによっては刺激されなかった。

実施例３：Ｅｐｏ反応性および抗Ｅｐｏ抗体による増殖阻害

実施例１で樹立した細胞株ＡＳ－Ｅ２のＥｐｏ反応性（増殖活性）および抗Ｅｐｏ抗体による増殖活性を試験した。

【００３０】ＡＳ－Ｅ２細胞１×１０^{5}細胞／ｍｌを用いて、（１）Ｅｐｏを添加しないで培養、（２）Ｅｐｏ ２ｕ／ｍｌを添加して培養、および（３）Ｅｐｏ ２ｕ／ｍｌと抗Ｅｐｏ抗体（αＥｐｏ：１／１００に希釈して使用）を添加して培養し、経時的（２、４、６、８日）に細胞数を顕微鏡下でカウントした。得られた結果を図２に示す。図から明らかなように、Ｅｐｏ添加した細胞はＥｐｏ添加しない細胞と比較して細胞数の顕著な増加が観察された。また、抗Ｅｐｏ抗体を同時に添加した場合には、この増殖活性が阻害された。

【００３１】次いで、上記３群の細胞をトリパンブルーで染色して生細胞数をカウントした結果を図３に示す。図から明らかなように、Ｅｐｏ添加しない細胞、およびＥｐｏと抗Ｅｐｏ抗体を添加した細胞では生細胞数が経時的に減少していたが、Ｅｐｏ添加した細胞では細胞数としてカウントされたほとんどが生細胞数としてもカウントされた。

実施例４：Ｅｐｏ受容体の発現の確認

ＡＳ－Ｅ２細胞上でＥｐｏ受容体が発現していることを以下の方法で確認した。

【００３２】ＡＳ－Ｅ２細胞を２４時間Ｅｐｏ非存在下で培養し、１×１０^{6}個の細胞に対して^{125} Ｉ－Ｅｐｏ（Ａｍｅｒｓｈａｍ社製）を０．０６～３ｎＭ添加して１５℃、３時間の条件で結合アッセイを行った。非特異的結合は、^{125} Ｉ－Ｅｐｏの４００倍量の非標識Ｅｐｏ存在下で測定した。なお、結合アッセイ培地の組成は、２％ ＢＳＡ、０．２％ ＮａＮ$_3$を含むＩＭＤＭ培地である。得られた結合を図４に示す。これより、ＡＳ－Ｅ２細胞上にＥｐｏ受容体が発現していることが確認された。

【００３３】次いで結合アッセイから得られたデータからＳｃａｔｃｈａｒｄ分析を行ってＥｐｏ受容体の解離定数（Ｋｄ値）と受容体数とを求めたところ、ＡＳ－Ｅ２細胞が発現しているＥｐｏ受容体が低親和性型１種類のみであることが示された。

実施例５：Ｊａｋ２、Ｅｐｏ受容体のリン酸化

ＡＳ－Ｅ２におけるＪａｋ２およびＥｐｏ受容体のリン酸化を以下の方法で試験してＥｐｏのシグナル伝達経路を検討した。

【００３４】ＡＳ－Ｅ２をＥｐｏ非存在下で２４時間培養し、次いでＥｐｏ（０または１０ｕ／ｍｌ）を添加して５分間反応させた後、１％ ＮＰ－４０（ナカライテスク株式会社製）、１０％ グリセロール、１ｍＭ ＰＭＳＦ（和光純薬工業株式会社製）、０．１ｍｇ／ｍｌ ロイペプチン（和光純薬工業株式会社製）、５μＭペプスタチンＡ（和光純薬工業株式会社製）、１ｍＭ ＥＤＴＡ、２ｍＭ オルトバナジウム酸ナトリウム（ＳＩＧＭＡ社製）を含むトリス緩衝塩溶液で細胞を可溶化した。これを用いて抗Ｊａｋ２抗体または抗Ｅｐｏ受容体抗体（いずれもＵＰＳＴＡＴＥ ＢＩＯＴＥＣＨＮＯＬＯＧＹ，ＩＮＣ．（ＵＳＡ）社製）で免疫沈降を行った。沈降物をＳＤＳ－ＰＡＧＥに付した後、ＰＶＤＦ膜に転写し、抗リン酸化チロシン抗体（和光純薬株式会社製）でチロシンリン酸化タンパク質を検出した。得られた結果を図５（Ａ：抗Ｊａｋ２抗体、Ｂ：抗Ｅｐｏ受容体抗体、また、Ａ、Ｂいずれにおいても、レーン１はＥｐｏ非添加の場合を、レーン２はＥｐｏ添加した場合を表す）に示す。図から明らかなように、ＡＳ－Ｅ２はＥｐｏに反応し、かつＥｐｏシグナル伝達系であるＪａｋ２およびＥｐｏ受容体のチロシンリン酸化が認められた。

実施例６：ＧＡＴＡ－１およびＧＡＴＡ－２のｍＲＮＡの発現

ＡＳ－Ｅ２におけるＧＡＴＡ－１およびＧＡＴＡ－２のｍＲＮＡの発現を以下のノーザンブロット分析により検討した。

【００３５】本発明の細胞株ＡＳ－Ｅ２、ならびに対照としてＫ５６２細胞株（慢性骨髄性白血病急性転化細胞株）およびＨＬ－６０細胞株（急性白血病細胞株）から、ＩＳＯＧＥＮ（ニッポンジーン社製）を用いて、全ＲＮＡをキット添付の処方に従って調製した。次いで、ノーザンブロット分析（Molecular Cloning: A Laboratory Manual, Sambrook et al., Cold Spring Harbor Laboratory Press, 1989）を行った。プローブの標識はＲａｎｄｏｍ Ｐｒｉｍｅｒ ＬａｂｅｌｌｉｎｇＫｉｔ（Ｔａｋａｒａ社製）を用いて行った。すなわち、ＰＶＣベクターのＥｃｏ ＲＩ部位に挿入されたＧＡＴＡ－１（Zon et al., Proc. Natl. Acad. Sci. USA 88:10638-10641, 1991）とＧＡＴＡ－２（Nagai et al., Blood 84:1074-1084, 1994）をコーディングするインサートを切り出し、キット添付の処方に従って標識プローブを作製した。

【００３６】前述の各細胞からの全ＲＮＡを１レーン当

たり３０μｇ使用し、アガロースゲル電気泳動を行った後、ハイブリダイゼーション・トランスファー・メンブレンとしてＨｙｂｏｎｄ－Ｎ（Ａｍｅｒｓｈａｍ社製）を使用し、キャピラリー法によりブロッティングした。ハイブリダイゼーションは５０％　ホルムアミド、５×ＳＳＰＥ、２×Ｄｅｎｈａｒｄｔ’ｓ　ｓｏｌｕｔｉｏｎ、０．１％　ＳＤＳの組成よりなる溶液を用いて４２℃で一晩実施した。ハイブリダイゼーション後、メンブレンを２×ＳＳＣ、０．１％　ＳＤＳで室温にて４回洗浄し、さらにＢＡＳ（Ｆｕｊｉ社製）にてオートラジオグラフィーを行い、画像解析をした。その結果を図６（Ａ：ＧＡＴＡ－１、Ｂ：ＧＡＴＡ－２、また、Ａ、Ｂいずれもにレーン１はＡＳ－Ｅ２、レーン２はＫ５６２、レーン３はＨＬ－６０を表す）に示す。図から明らかなように、ＧＡＴＡ－１はＡＳ－Ｅ２とＫ５６２に明瞭なバンドとして検出され、またＧＡＴＡ－２はＡＳ－Ｅ２、Ｋ５６２、ＨＬ－６０のいずれの細胞株でも認められた。

【００３７】

【発明の効果】本発明によってエリスロポエチンのみに特異的に依存して増殖し、エリスロポエチン活性のスクリーニングに用い得る細胞株の樹立が達成された。本発明の細胞株を用いてエリスロポエチン活性を有すると推定される化合物のエリスロポエチン活性を測定するインビトロスクリーニング法が可能となった。このインビトロスクリーニングは本発明の細胞株の細胞増殖または分化誘導を試験することにより実施できる。また、この試験法によってＥｐｏ濃度を測定することも可能である。さらに、本発明の細胞株を、初期赤芽球白血病の病態解析や治療モデルとして使用することができる。さらには、本発明の細胞株を用いて、エリスロポエチンに特異的なシグナル伝達タンパク質／遺伝子を解明するための実験を行うことができる。

【００３８】これらの点から本発明のエリスロポエチン依存性ヒト細胞株の利用価値は極めて大きい。

【図面の簡単な説明】

【図１】ＡＳ－Ｅ２細胞に対する各種サイトカインの反応性を示す図である。

【図２】ＡＳ－Ｅ２のＥｐｏ反応性および抗Ｅｐｏ抗体による増殖活性を示すための、顕微鏡で観察した経時的な細胞数を示す図である。（●）はＥｐｏ　２ｕ／ｍｌを添加した細胞、（△）はＥｐｏを添加しない細胞、（□）はＥｐｏ　２ｕ／ｍｌと抗Ｅｐｏ抗体（αＥｐｏ：１／１００希釈）を添加した細胞を表す。

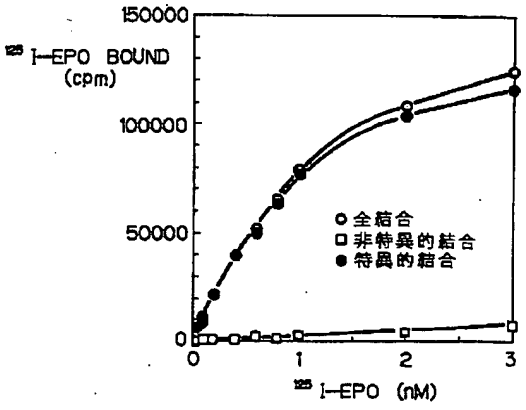
【図３】ＡＳ－Ｅ２のＥｐｏ反応性および抗Ｅｐｏ抗体による増殖活性を示すための、トリパンブルー染色した経時的な生細胞数を示す図である。（●）はＥｐｏ　２ｕ／ｍｌを添加した細胞、（△）はＥｐｏを添加しない細胞、（□）はＥｐｏ２ｕ／ｍｌと抗Ｅｐｏ抗体（αＥｐｏ：１／１００希釈）を添加した細胞を表す。

【図４】^{125}Ｉ－Ｅｐｏを用いるＡＳ－Ｅ２の結合アッセイを示す図である。（○）は全結合活性、（□）は非特異的結合活性、（●）は特異的結合活性を表す。

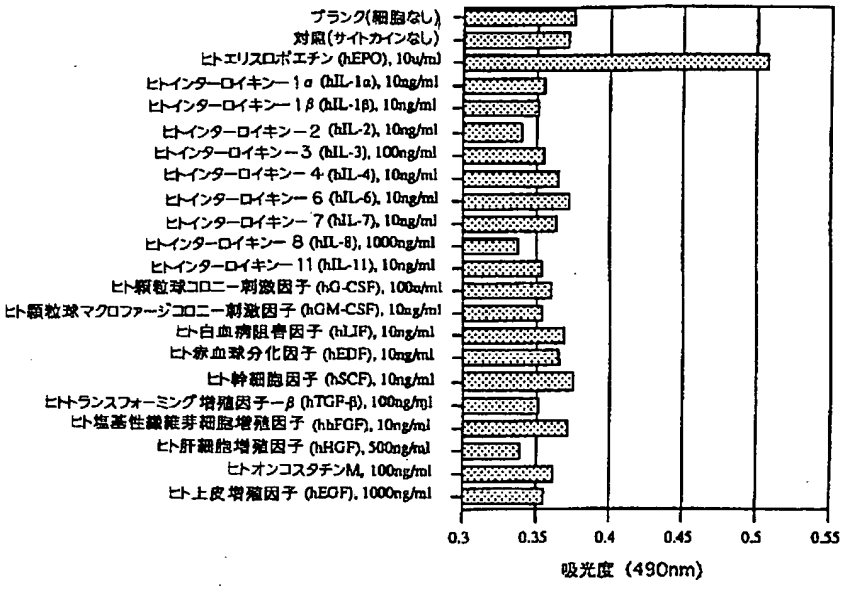
【図５】ＡＳ－Ｅ２におけるＪａｋ２およびＥｐｏ受容体のリン酸化を示す図（電気泳動の写真）である。図中、Ａは抗Ｊａｋ２抗体（レーン１はＥｐｏ非添加；レーン２はＥｐｏ添加）、Ｂは抗Ｅｐｏ受容体抗体（レーン１はＥｐｏ非添加；レーン２はＥｐｏ添加）との免疫沈降物をＳＤＳ－ＰＡＧＥの電気泳動に付し、ＰＶＤＦ膜に転写し、抗リン酸化チロシン抗体でチロシンリン酸化タンパク質を検出した図を表す。

【図６】ＡＳ－Ｅ２、Ｋ５６２、ＨＬ－６０細胞株におけるＧＡＴＡ－１、ＧＡＴＡ－２のｍＲＮＡの発現を示す図（電気泳動の写真）である。図中、ＡはＧＡＴＡ－１（レーン１はＡＳ－Ｅ２；レーン２はＫ５６２；レーン３はＨＬ－６０）、ＢはＧＡＴＡ－２（レーン１はＡＳ－Ｅ２；レーン２はＫ５６２；レーン３はＨＬ－６０）をプローブとして用いたノーザンブロットの結果を表す。

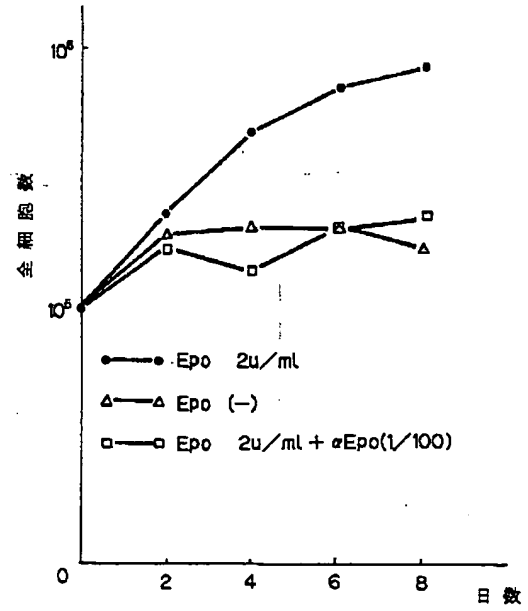
【図４】



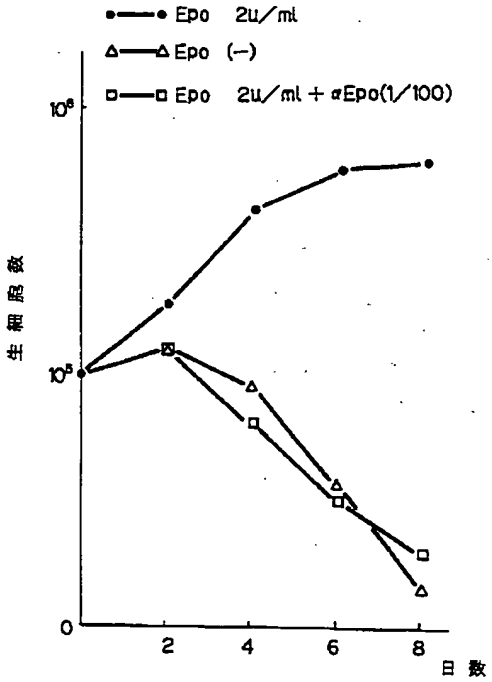
【図１】


ブランク(細胞なし)
対照(サイトカインなし)
ヒトエリスロポエチン (hEPO), 10u/ml
ヒトインターロイキン－1α (hIL-1α), 10ng/ml
ヒトインターロイキン－1β (hIL-1β), 10ng/ml
ヒトインターロイキン－2 (hIL-2), 10ng/ml
ヒトインターロイキン－3 (hIL-3), 100ng/ml
ヒトインターロイキン－4 (hIL-4), 10ng/ml
ヒトインターロイキン－6 (hIL-6), 10ng/ml
ヒトインターロイキン－7 (hIL-7), 10ng/ml
ヒトインターロイキン－8 (hIL-8), 1000ng/ml
ヒトインターロイキン－11 (hIL-11), 10ng/ml
ヒト顆粒球コロニー刺激因子 (hG-CSF), 100ng/ml
ヒト顆粒球マクロファージコロニー刺激因子 (hGM-CSF), 10ng/ml
ヒト白血病阻害因子 (hLIF), 10ng/ml
ヒト赤血球分化因子 (hEDF), 10ng/ml
ヒト幹細胞因子 (hSCF), 10ng/ml
ヒトトランスフォーミング増殖因子－β (hTGF-β), 100ng/ml
ヒト塩基性繊維芽細胞増殖因子 (hbFGF), 10ng/ml
ヒト肝細胞増殖因子 (hHGF), 500ng/ml
ヒトオンコスタチンM, 100ng/ml
ヒト上皮増殖因子 (hEGF), 1000ng/ml
0.3
0.35
0.4
0.45
0.5
0.55
吸光度 (490nm)

【図２】

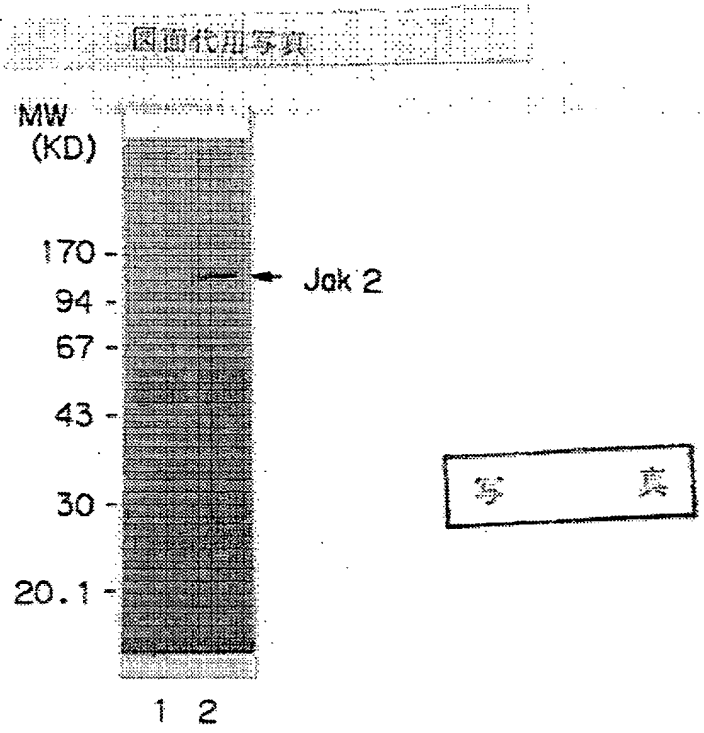

10^6
10^5
0
2
4
6
8
日 数
生細胞数
Epo 2u/ml
Epo (−)
Epo 2u/ml + αEpo(1/100)

【図３】

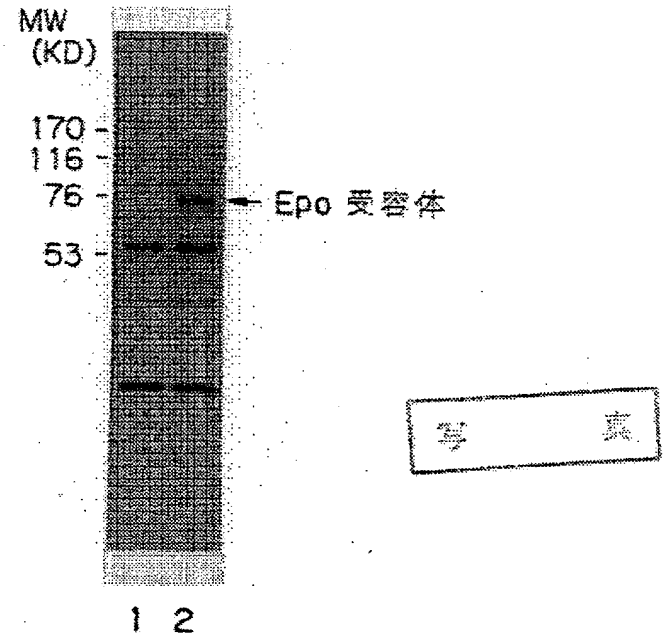

Epo 2u/ml
Epo (−)
Epo 2u/ml + αEpo(1/100)
10^6
10^5
0
2
4
6
8
日 数
生細胞数

【図5】

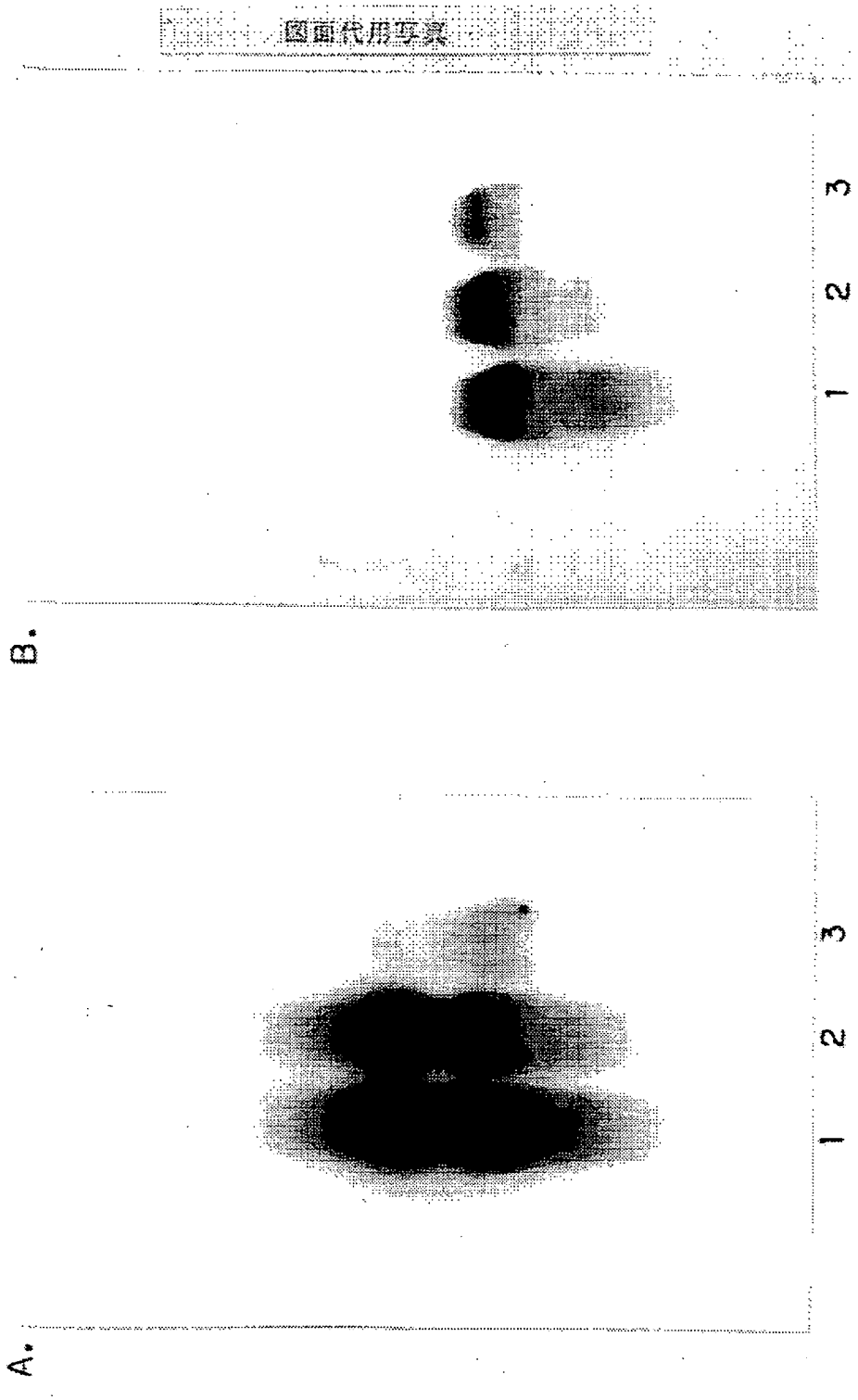
A.


図面代用写真
MW
(KD)
170
94
67
43
30
20.1
Jak 2
写 真
1 2

B.


MW
(KD)
170
116
76
53
Epo 受容体
写 真
1 2

【図６】



フロントページの続き

(51) Int.Cl.[6]	識別記号	庁内整理番号	F I		技術表示箇所
Ｇ０１Ｎ　33/53		V			
		K			
//(Ｃ１２Ｎ　5/06					
Ｃ１２Ｒ　1:91)					
			Ａ６１Ｋ　37/02	ＡＣＣ	
			(Ｃ１２Ｎ　5/00		E
			Ｃ１２Ｒ　1:91)		